Angiotensin-Converting Enzyme Inhibition Prevents the Release of Monocytes From Their Splenic Reservoir in Mice With Myocardial Infarction

Florian Leuschner, Peter Panizzi, Isabel Chico-Calero, Won Woo Lee, Takuya Ueno, Virna Cortez-Retamozo, Peter Waterman, Rostic Gorbatov, Brett Marinelli, Yoshiko Iwamoto, Aleksey Chudnovskiy, Jose-Luiz Figueiredo, David E. Sosnovik, Mikael J. Pittet, Filip K. Swirski, Ralph Weissleder, Matthias Nahrendorf

Rationale: Monocytes recruited to ischemic myocardium originate from a reservoir in the spleen, and the release from their splenic niche relies on angiotensin (Ang) II signaling.

Objective: Because monocytes are centrally involved in tissue repair after ischemia, we hypothesized that early angiotensin-converting enzyme (ACE) inhibitor therapy impacts healing after myocardial infarction partly via effects on monocyte traffic.

Methods and Results: In a mouse model of permanent coronary ligation, enalapril arrested the release of monocytes from the splenic reservoir and consequently reduced their recruitment into the healing infarct by 45%, as quantified by flow cytometry of digested infarcts. Time-lapse intravital microscopy revealed that enalapril reduces monocyte motility in the spleen. In vitro migration assays and Western blotting showed that this was caused by reduced signaling through the Ang II type 1 receptor. We then studied the long-term consequences of blocked splenic monocyte release in atherosclerotic apolipoprotein (apo)E−/− mice, in which infarct healing is impaired because of excessive inflammation in the cardiac wound. Enalapril improved histologic healing biomarkers and reduced inflammation in infarcts measured by FMT-CT (fluorescence molecular tomography in conjunction with x-ray computed tomography) of proteolytic activity. ACE inhibition improved MRI-derived ejection fraction by 14% on day 21, despite initially comparable infarct size. In apoE−/− mice, ischemia/reperfusion injury resulted in larger infarct size and enhanced monocyte recruitment and was reversible by enalapril treatment. Splenectomy reproduced antiinflammatory effects of enalapril.

Conclusion: This study suggests that benefits of early ACE inhibition after myocardial infarction can partially be attributed to its potent antiinflammatory impact on the splenic monocyte reservoir. (Circ Res. 2010;107:1364-1373.)

Key Words: monocyte ■ spleen ■ ACE inhibitor ■ myocardial infarction ■ heart failure

Angiotensin-converting enzyme (ACE) inhibitors, which lower systemic and tissue levels of angiotensin (Ang) II by reducing cleavage of the C-terminal dipeptide from Ang-1, were developed as antihypertensive agents. They have evolved into a mainstay of heart failure therapy; current guidelines recommend initiation of therapy early after myocardial infarction (MI),1 and are based on clinical trials showing that ACE inhibition decreases mortality.2 Initially, these effects were attributed to hemodynamic unloading of the left ventricle.3 Subsequently, it became clear that ACE inhibitors also decrease hypertrophy and fibrosis through direct effects on heart tissue.4 These well-described effects reduce left ventricular remodeling following MI, and therapy is therefore continued indefinitely.

The effect of ACE inhibitors on the monocyte response during the first week after MI is less well understood. There is emerging evidence that Ang II, which sharply increases in circulation after MI, is a proinflammatory peptide that closely interacts with the immune system, including monocytes.4–7 Furthermore, Ang II can modulate the regional cytokine milieu.8–11 It has recently been shown that 40% to 70% of monocytes acutely recruited to the infarct originate from a splenic reservoir.6 In the steady state, monocytes reside in clusters of ∼50 cells in the subcapsular red pulp of the spleen. On interaction of Ang II with its cell surface receptor, Ang II type 1 receptor (AT1R), splenic monocytes increase their motility and intravasate into nearby splenic veins. In the mouse, this “emergency reservoir” releases up to 1 million...
monocytes within 24 hours after MI, which are subsequently recruited into the infarct mainly via interaction of the chemokine MCP-1 with its cognate receptor CCR2. The cells then assume a central role in orchestrating the healing wound. They promote removal of cell debris and extracellular matrix through proteolysis and phagocytosis and regulate tissue repair by stimulating angiogenesis and fibroblasts. However, an excessive inflammatory response is deleterious. In atherosclerotic apolipoprotein (apo)E−/− mice with coronary ligation, the coexisting systemic inflammation associated with hyperlipidemia increases levels of circulating monocytes and their recruitment to the infarct. Consequently, scar healing is compromised and development of heart failure accentuated. In patients, increased levels of blood monocytes during MI also correlate with enhanced left ventricular dilation and adverse outcome.

Here, we hypothesized that ACE inhibition reduces splenic release of monocytes, decreases their recruitment into the infarct, curbs excessive infarct inflammation, and promotes coordinated wound healing. We further propose that the effect of ACE inhibition on the innate immune system presents a new mechanism that at least partially explains the reduction of heart failure following MI.

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Animal Models**

Female C57BL/6J and apoE−/− mice were purchased from Jackson Laboratory. Cx3cr1GFP mice were obtained by breeding Cx3cr1GFP mice with C57BL/6J mice. Cx3cr1GFP−/− mice have one Cx3cr1 allele replaced with cDNA encoding eGFP and can be used to track monocytes. ApoE−/− mice had an average age of 45 weeks and were on a high-cholesterol diet. The institutional animal welfare committee approved the research reported.

MI, ischemia/reperfusion injury (IRI), and splenectomy were performed as described (see the Online Data Supplement). Mice were treated with a dose of 100 mg/kg enalapril daily for the acute studies (euthanasia 1 day after MI) or 20 mg/kg enalapril for the chronic studies. Treatment was initiated 2 days before MI and continued for 7 days thereafter. In additional cohorts, mice were splenectomized at the time of coronary ligation. Hydralazine was given at a dose of 15 mg/kg daily. Treatment was performed by gastric gavage.

**Histology**

Histology of spleens and hearts was assessed as described in the Online Methods.

**Flow Cytometry**

Mice were euthanized on days 1, 2, 3, and 5 after MI and 1 day after IRI for flow cytometry (n = 3 to 5 mice per group and time point). Spleens, hearts, and blood were prepared and analyzed for total organ monocyte numbers by multicolor flow cytometry.

**ELISA for Serum Ang II Levels**

Ang II concentration in blood was determined with an Ang II ELISA.

**Intravital Microscopy**

Intravital microscopy of splenic monocytes was performed in Cx3cr1GFP−/− mice. During isoflurane anesthesia, the spleen was exteriorized and imaged with a prototypic intravital laser scanning microscope (IV100, Olympus Corporation).

**Non-standard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ACE</td>
<td>angiotensin-converting enzyme</td>
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<td>Ang II</td>
<td>angiotensin II</td>
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<td>apoE</td>
<td>apolipoprotein E</td>
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<td>AT1,R</td>
<td>Ang II type 1 receptor</td>
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<td>FMT-CT</td>
<td>fluorescence molecular tomography in conjunction with x-ray computed tomography</td>
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<td>IRI</td>
<td>ischemia/reperfusion injury</td>
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<td>IVM</td>
<td>intravital microscopy</td>
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<td>MI</td>
<td>myocardial infarction</td>
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<td>TTC</td>
<td>triphenyltetrazolium chloride</td>
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**In Vitro Migration**

Migration experiments using Ang II as a chemoattractant were performed using sorted monocytes from spleen. For the receptor blocking condition, cells were pretreated with the AT1,R antagonist losartan.

**Western for AT1,R on Splenic Monocytes**

Splenic monocytes were isolated by flow-activated cell sorting and dimerization of the AT1,R was assessed by Western blotting.

**Quantitative PCR**

Infarct tissue was examined for expression of markers of inflammatory monocytes (Ly-6C, differentiated macrophages (CD68; Mac3), TNF-α, TGF-β, myeloperoxidase, and appropriate controls (GAPDH). TGF-β mRNA was also measured in monocytes isolated from the heart.

**Fluorescence Molecular Tomography–X-Ray Computed Tomography**

On day 1 after MI, we performed fluorescence molecular tomography (FMT-CT) fluorescence molecular tomography in conjunction with x-ray computed tomography imaging to interrogate the magnitude of inflammation 24 hours after injection of 5 nmol of a pan-cathepsin protease sensor (Prosense-680, PerkinElmer) in apoE−/− mice with and without enalapril treatment and apoE−/− mice that were splenectomized at the time of coronary ligation.

**Magnetic Resonance Imaging**

We performed in vivo MRI on day 21 after MI in apoE−/− mice with and without enalapril treatment and in apoE−/− mice that were splenectomized at the time of coronary ligation.

**Ischemia/Reperfusion Injury**

IRI was studied in wild-type mice, apoE−/− with and without enalapril treatment, and in apoE−/− that were splenectomized immediately before ischemia. The area at risk was assessed using fluorescent microspheres, and the resulting infarct size by triphenyltetrazolium chloride (TTC) staining.

**Blood Pressure Measurements**

Blood pressure was monitored using the noninvasive tail cuff method.

**Statistics**

Results are expressed as means ± SEM. Statistical comparisons between 2 groups were evaluated by Student t test and corrected by ANOVA for multiple comparisons. A value of P < 0.05 was considered statistically significant.
ACE Inhibition Prevents the Release of Splenic Monocytes in Acute MI

Myocardial injury or administration of Ang II results in increased motility of monocytes in the spleen. We therefore investigated whether lowering levels of Ang II through ACE inhibition impacts splenic monocytes. First, we assessed monocyte numbers in the spleen on day 1 after permanent coronary ligation with and without enalapril treatment. The subcapsular region is the site where monocytes are stored in the spleen. Immunofluorescence staining for CD11b showed significant reduction of CD11b+ cells in the subcapsular region after MI. Treatment with enalapril completely abolished splenic loss of monocytes after MI (P<0.0001; Figure 1A).

To quantify total monocyte numbers in the whole spleen, we used flow cytometry 1 day after coronary ligation. MI induced massive release of monocytes from the spleen. Monocyte numbers in spleens of mice with MI were reduced by 42% compared with spleens from naive mice (Figure 1B; P<0.01). In contrast, infected mice treated with enalapril showed practically no release of splenic monocytes (no MI, 1.24×10^6 monocytes per spleen; MI plus enalapril, 1.22×10^6 monocytes per spleen; P=NS; Figure 1B). The effect was observed for both subsets, Ly6-Chigh as well as Ly6-Clow monocytes.

Next, we investigated if the effect on splenic monocytes was related to vasodilatory properties of ACE inhibitors. We confirmed that enalapril significantly reduced Ang II serum levels in plasma (P<0.05; Figure 1C). We then treated mice with the direct vasodilator Hydralazine and chose a dose of 15 mg/kg/d, which had previously been shown to reduce systolic blood pressure to <80 mm Hg. Flow cytometric analysis of the spleen showed that Hydralazine did not reduce the release of monocytes after MI (P=NS versus untreated MI; Figure 1D). We thus concluded that monocyte retention in the spleen by enalapril is likely not related to its vasodilatory properties, because lowering of blood pressure with a direct vasodilator did not reproduce its effect. Blood pressure data in various cohorts are shown in Online Table I.

ACE Inhibition Reduces the Motility of Splenic Monocytes

Next, we aimed to investigate how ACE inhibition reduces monocyte release from the spleen. We used time-lapse intravital microscopy, a technique that allows to track endogenous monocytes in the subcapsular red pulp of the spleen in live animals. We investigated the spleens of Cx3cr1^GFP+ mice, in which 1 Cx3cr1 allele is replaced with cDNA encoding eGFP. Thus, cells that express the fractalkine receptor are rendered GFP+ and can be followed with fluorescence imaging. Within the splenic capsule, 95% of the GFP+ cell population consists of monocytes, macrophages and dendritic cells. The present study confirmed that splenic monocytes increase motility after MI (4-fold increase, P<0.01; Figure 2A and Online Movie I). Previously, it was shown that this increase in motility leads to intravasation into sinusoids and spleen veins and departure of the cells from the organ. When treated with enalapril, monocytes did not increase motility after MI (Figure 2A and Online Movie I). Splenic macrophages or dendritic cells showed very low displacement and there was no difference between both groups, as expected (Figure 2A).

In vitro cell migration experiments corroborated the importance of Ang II signaling for monocyte motility. Ang II induced rigorous migration of monocytes isolated from the spleen; however, cotreatment with the AT1R antagonist losartan reduced this effect significantly (Figure 2B), suggesting that mobilization of splenic monocytes requires Ang II/AT1R signaling.

Next, we investigated dimeric forms of the AT1 receptor on monocytes sorted from spleens by Western blot. Ang II/AT1R signaling requires dimerization and covalent crosslinking of the receptor, an event that can be quantified by Western blot using reduced gels. We found that MI triggers AT1R dimerization in splenic monocytes. However, enalapril reduced this effect (Figure 2C). These data describe the mechanism how ACE inhibition blocks splenic monocyte release: they indicate that ACE inhibition reduces Ang II/AT1R signaling in monocytes, which directly affects monocyte motility, and thus departure from the splenic reservoir.
Blocking of the Splenic Monocyte Release
Diminishes Recruitment to the Infarct

Reduced mobilization of splenic monocytes by enalapril should decrease accumulation of these cells in ischemic myocardium. Flow cytometry confirmed a marked reduction in the number of monocytes accumulating in the infarct on day 1, 3, and 5 after permanent coronary ligation (Figure 3A). The inflammatory response on day 1 after MI is dominated by Ly-6Chigh monocytes.18 Accordingly, the impact of enalapril was more profound on this subset (Figure 3A). However, Ly-6Clow monocyte numbers were also reduced in the heart, especially at later time points (Figure 3A). Immunoreactive staining for CD11b and MAC-3 confirmed that enalapril reduced the number of myeloid cells in 1-day-old infarcts (Figure 3B). The number of CD11b+ cells per high power field was reduced from 52±4 to 31±4 (P<0.01), and the area positive for MAC-3 from 5.8±0.7 to 3.5±0.4% (P<0.01).

Because some patients will not be receiving ACE inhibitor therapy before their first MI, we next tested the effects of enalapril 1 hour or 24 hours after coronary ligation. This experiment models the situation of a first MI, when treatment is initiated early after ischemia. The impact of ACE inhibition on monocyte flux was preserved, but somewhat attenuated when started with a 24-hour delay, probably because there was a period of uninhibited monocyte release after MI (Online Figure I).

Quantitative PCR of infarct tissue showed that enalapril profoundly reduced expression of TGF-β, myeloperoxidase, and TNF-α (Figure 4). MAC-3, CD68, and Ly-6C, which are genes primarily expressed by inflammatory monocytes and macrophages, were also reduced (Figure 4). Furthermore, we assessed the mRNA level of TGF-β in monocytes that were isolated by flow-activated cell sorting and found levels that were 4.3-fold higher than in infarct tissue (P<0.01 versus infarct tissue). Thus, we concluded that monocytes may be a source of TGF-β after MI.
ACE Inhibition Reduces Inflammation in Infarcts of ApoE−/− Mice

We next tested whether inhibiting the mobilization of the splenic monocyte reservoir affects cardiac wound healing. We chose to investigate infarct healing in apoE−/− mice, because their heightened systemic inflammation, specifically the increased availability of monocytes in circulation, may resemble patients with atherosclerosis better.17 This model is particularly helpful when innate immune responses to ischemia and tissue repair are studied.14 Flow cytometric analysis of apoE−/− mice on day 1 after permanent coronary ligation showed that enalapril prevented the release of splenic monocytes and reduced blood monocytopsis (Figure 5). Notably, monocyte numbers in the infarct were reduced to levels seen in wild-type mice (wild type, 0.42×10⁶±0.1×10⁶; apoE−/−, 1.23×10⁶±0.1×10⁶; apoE−/− enalapril, 0.35×10⁶±0.1×10⁶). The treatment reduced the number of both Ly-6Chigh and Ly-6Clow monocytes (Figure 5B).

Wound healing was then assessed by histology on days 4, 7, and 10 after coronary ligation. Immunoreactive staining of the infarcted tissue from apoE−/− mice showed that enalapril supported the resolution of inflammation, because numbers of inflammatory cells, including monocytes, neutrophils, and macrophages were reduced (Figure 6). We had previously found that the infarcts of apoE−/− mice contain more microvessels and less collagen, likely because of increased delivery of proangiogenic factors and proteases by monocytes.18 This was reversed in the present study: enalapril decreased the number of new vessels and augmented collagen content in the border zone (Figure 6). Thus, reduction of inflammation by enalapril improved infarct healing in apoE−/− mice.

Early ACE Inhibitor Treatment Reduces Protease Activity and Improves Left Ventricular Remodeling in ApoE−/− Mice With MI

We next evaluated the effects of improved healing in enalapril-treated mice on left ventricular remodeling. Infarct size and proteolytic activity were measured by FMT-CT17 on day 1 after MI, followed by MRI on day 21 to evaluate left ventricular anatomy and function. A group of apoE−/− mice received 20 mg/kg enalapril (see flow chart in Figure 7) and was compared with untreated apoE−/− mice. Importantly,
treatment was terminated on day 7 to study ACE inhibitor effects during the early healing phase after MI. CT imaging found that infarct size on day 1 was similar between study groups. To assess infarct inflammation in vivo, we quanti-
tated proteolytic activity in infarcts using an activatable fluorescent protease sensor and FMT-CT.17,30 Regions of interest were identified in the FMT dataset based on anatomic information provided by fusion with CT images. In line with flow cytometric results showing lower monocyte numbers, enalapril reduced proteolytic activity in the infarct (Figure 7; P<0.05). Enalapril therapy was discontinued on day 7, when inflammation in murine infarcts subsides and actions of the ACE inhibitor on the remote myocardium would predomi-
nate. Mice were then followed up on day 21 after MI by MRI volumetry. Untreated mice showed substantial left ventricular remodeling and a reduced ejection fraction. However, MRI parameters improved in the enalapril-treated cohort. These mice had higher ejection fractions and smaller end-diastolic volumes (Figure 7; P<0.05). Increased scar thickness (Figure 7; P<0.05) indicated that the treatment had influenced infarct healing, as infarct expansion and thinning is a hallmark of impaired healing.13 Left ventricular mass was also reduced (Figure 7).

When enalapril treatment was started 1 hour after coronary ligation, the strong antiinflammatory effects seen by FMT-CT were preserved (protease sensor activation: MI 67.6±17.0 pmol, MI enalapril 26.2±8.2 pmol, P<0.05). In the same mice, MRI derived ejection fraction on day 21 was higher when compared with untreated mice (control MI, 34±4%, MI enalapril 43±2%, P<0.05).

Early ACE inhibitor treatment has beneficial effects on left ventricular hemodynamics, and reduces local ACE activity in the heart. We wanted to explore to what extent the impact on the monocyte flux contributes to the overall benefits of enalapril. We thus neutralized the splenic monocyte reservoir by surgical removal of the spleen at the time of coronary ligation. As in enalapril-treated mice, this results in a reduced availability of splenic monocytes, but leaves nonspleen targets of enalapril untouched. Hence, this cohort models the isolated splenic effect of ACE inhibition. FMT-CT showed a similar reduction of protease activity in the infarct of apoE<sup>−/−</sup> mice by enalapril and splenectomy (Figure 7; P<0.05). This is in line with the notion that both procedures reduce the recruitment of monocytes into the infarct, cells that are the major source of proteases in MI.31 In apoE<sup>−/−</sup> mice, splenec-
tomy had a beneficial effect on left ventricular remodeling, albeit to a lesser degree than treatment with enalapril (Figure 7; P<0.05). Compared with untreated apoE<sup>−/−</sup> mice, the EF was improved by 9% in splenectomized mice, and by 16% in mice treated with enalapril. Similar trends were observed for the end-diastolic volume, left ventricular mass, and scar thickness, all measured by MRI (Figure 7).

IRI Is Increased in ApoE<sup>−/−</sup> Mice and Ameliorated by Enalapril and by Splenectomy

Most patients with acute MI reach the hospital in time for reperfusion therapy. We therefore investigated the role of monocytes in IRI. During 35 minutes of ischemia, mice were injected with fluorescent microspheres to delineate the area at risk. Twenty-four hours after reperfusion, infarct size was determined by TTC staining, and area at risk by fluorescence imaging of myocardial rings (Figure 8A). Compared with wild-type mice, the infarct/area at risk ratio was profoundly increased in apoE<sup>−/−</sup> mice (Figure 8B), and this was paralleled by an increased number of monocytes recruited to the injured myocardium (Figure 8C and 8D). Treatment with enalapril and surgical removal of the splenic monocyte reservoir reduced the infarct/area at risk ratio in a similar fashion. Likewise, the number of monocytes recruited to the heart was reduced in parallel by enalapril treatment and splenectomy (Figure 8C and 8D). Analysis of spleens revealed that enalapril inhibited the release of the splenic monocyte reservoir following IRI (Figure 8E).
Discussion

In 2010, an estimated 785,000 Americans will have a new acute coronary syndrome, 1 every 25 seconds. Most of these patients will receive ACE inhibitor therapy within 24 hours. Here, we identified a new mechanism that contributes to the beneficial effects of early ACE inhibitor therapy after MI. In mice with coronary ligation, enalapril decreased Ang II/AT1R signaling on monocytes in the spleen and thus inhibited their massive mobilization from the splenic reservoir. Preventing the release of splenic monocytes subsequently controlled their recruitment in infarcts, and short-term treatment during the wound-healing phase after MI had a profound long-term impact on left ventricular remodeling.

Coronary ligation in apoE−/− mice allows the study of MI in the context of preexisting chronic inflammation. In these mice, hyperlipidemia induces a progressive increase of the monocyte pool. The cells are recruited into the vessel wall, a process that is now well understood to drive atherosclerotic lesion formation and progression. We chose this model because it likely reproduces innate immune processes during acute coronary syndrome, 1 every 25 seconds. Most of these patients will receive ACE inhibitor therapy within 24 hours.

Mice with atherosclerosis or hypercholesterolemia, but induced by LPS injections) causes excessive monocyte recruitment in infarcts, impaired infarct healing and accelerated left ventricular dilation. Of note, a parallel relationship of increased blood monocyte levels and left ventricular remodeling was recently described in patients after MI. In the present study, enalapril substantially reduced the mobilization of monocytes from the spleen and lowered their numbers in blood and infarct of wild-type, as well as apoE−/−, mice. Therefore, the antiinflammatory properties of ACE inhibition may mitigate the accentuated immune activity in patients with acute coronary syndromes and thus partly contribute to improving their prognosis.

Histological assessment of enalapril-treated apoE−/− mice after MI showed faster resolution of inflammation, and normalization of wound-healing parameters such as number of neovessels and collagen content in the border zone. Direct action of Ang II on fibroblasts promotes collagen synthesis and ACE inhibition reduces collagen content in the remote zone and scar. In the present study, we found increased collagen content in the scar of treated apoE−/− mice. Because enalapril drastically reduced numbers of inflammatory monocytes and protease activity, it likely changed the balance of matrix breakdown and synthesis. Increased scar thickness measured by MRI indicates that improved healing reduced infarct expansion, which then resulted in attenuated left ventricular remodeling and higher ejection fraction in enalapril-treated mice. Anatomic and functional MR data measured 3 weeks after MI were improved although therapy was limited to the first week, suggesting that treatment during the early period of infarct healing garners long-term benefits.

ACE inhibition has also been shown to modulate inflammatory cytokine expression, for instance TNF-α or TGF-β, which may influence left ventricular remodeling and heart failure. Because these regulators of innate immunity are found in monocytes, it is likely that the effects of ACE inhibition on monocyte traffic, at least partially, contribute to a change in the regional cytokine milieu. This is supported by the 4.3-fold higher TGF-β mRNA levels in monocytes when compared with infarct tissue levels.

Similar to previous reports which showed increased recruitment of monocytes to the infarct after permanent coronary artery occlusion in apoE−/− mice with atherosclerosis and blood monocytes, we found that monocyte recruitment is more than doubled after IRI in these mice. The majority of recruited cells belonged to the inflammatory Ly-6Chigh subset, which carry high payloads of potentially harmful inflammatory mediators. When compared with wild-type mice, the infarct size was increased in apoE−/− mice. Treatment with enalapril as well as splenectomy reduced the
number of recruited monocytes and the quantity of infarcted tissue to levels seen in wild-type mice. The lower number of monocytes in heart tissue was attributable to inhibited splenic release following ACE inhibitor treatment. These findings position monocytes as therapeutic targets in IRI, and confirm that ACE inhibitors reduce infarct size\(^{44-46}\) in a model that accounts for the heightened activity of the immune system in atherosclerosis.\(^{5,6}\)

ACE inhibitor therapy has many reported targets, including beneficial systemic hemodynamic effects and decrease of Ang II tissue levels.\(^{5}\) To study the relative contribution of splenic effects, we removed the splenic monocyte reservoir surgically in apoE\(^{-/-}\) mice. Infarct protease activity was reduced to the same extent by splenectomy and enalapril, which suggests dominance of the splenic effects in the acute antiinflammatory properties of enalapril. Ejection fraction on day 21 after MI was improved partially when compared with enalapril treatment (9% versus 16% improvement over untreated mice with MI). It is important to stress that these beneficial effects of splenectomy were observed in apoE\(^{-/-}\) mice, which recruit too many monocytes to the healing infarct.\(^{17}\) In healthy individuals, the existence of a rapidly deployable reservoir of myeloid cells is likely an evolutionary advantage because it allows the immune system to respond to injury quickly. The spleen may have many favorable effects for wound healing, and health in general. This is highlighted by a study of veterans who lost their spleens from World War II injuries and consequently had an increased cardiovascular mortality.\(^{57}\) Also, the wholesale removal of the organ may compromise other, not yet known, protective functions in infarct healing.

Current guidelines of the American College of Cardiology and the American Heart Association recommend that oral ACE inhibitor therapy should be initiated within the first 24 hours of suspected acute MI in patients without contraindications. If the observations of this study translate into humans, the treatment would also curb inflammation in patients with a raised level of immune activity attributable to coexisting atherosclerotic disease. The data presented here provide additional mechanistic insight into the mortality reduction by early ACE inhibitor treatment found in clinical studies (HEART,\(^{48}\) SMILE\(^{49}\)) and suggest that systemic or local infarct inflammation may be an important therapeutic target for guiding therapy in individual patients. The level of inflammation in the infarct could be monitored by monocyt/e macrophage imaging. To this end, the phagocytic or inflammatory properties of myeloid cells could be harnessed for MR imaging.\(^{50,51}\) Alternatively, as in this study, a key monocyte function could serve as an imaging biomarker. We used fluorescence molecular tomography to quantify protease-dependent activation of an optical beacon in the myocardium, which can be accomplished with nuclear imaging in patients.\(^{52}\) While these technologies have not yet been translated into clinical care, the level of circulating monocytes may offer an approximation for the number of cells in the infarct. However, this association needs to be confirmed in patients.

In conclusion, we show that ACE inhibitor treatment has a profound impact on the innate immune response after MI and that this newly discovered mechanism contributes substantially to the benefits of early ACE inhibitor therapy after MI. The inhibition of monocyte mobilization from their splenic reservoir represents a powerful antiinflammatory action that may have therapeutic implications beyond treatment of hypertension and heart failure.

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Disclosures

None.

References


What Is Known?

- Monocytes and macrophages are the most abundant host cells in acute cardiac infarcts and orchestrate the wound-healing process following ischemia. The type of healing process determines whether left ventricular remodeling and heart failure will ensue.
- One day after onset of ischemia, the majority of monocytes/macrophages found in the infarct have come from a splenic reservoir. There, monocytes reside in clusters in the subcapsular red pulp and are released into the circulation.
- Initiation of ACE inhibitor therapy after myocardial infarction (MI) is recommended in current guidelines. However, the impact of ACE inhibition on infarct inflammation is incompletely understood.

What New Information Does This Article Contribute?

- We show a new effect of ACE inhibitors on monocytes. Treatment with enalapril abolishes splenic monocyte release after coronary ligation or ischemia/reperfusion injury (IRI) in mice.
- Blockade of splenic monocyte release drastically reduces the number of recruited monocytes/macrophages to the heart and improves infarct healing in apoE$^{-/-}$ mice, a model system that takes into account the heightened systemic activity of innate immunity in patients with atherosclerosis.
- IRI is enhanced in apoE$^{-/-}$ mice with inflammatory monocytes. The increase of infarct size is reversed by enalapril treatment, which reduces the number of inflammatory cells in the ischemic area by blocking splenic monocyte release.

After myocardial infarction, millions of monocytes and their lineage-descendent macrophages are rapidly recruited to orchestrate removal of debris and formation of granulation tissue. Because these cells govern key aspects of wound healing, e.g., neovessel formation and the buildup of new extracellular matrix by fibroblasts, they have a profound impact on the repair process. Excessive, as well as insufficient, infarct inflammation may lead to infarct rupture or expansion, and these early events set the stage for left ventricular remodeling. Myocardial infarction is a prominent cause of heart failure. Hence, monocyte/macrophages are emerging as therapeutic targets to prevent post-MI remodeling. In our present work, we describe that treatment with the ACE inhibitor enalapril abolishes the release of monocytes from their splenic reservoir. Consequently, the number of monocytes in the infarct is drastically reduced. In apoE$^{-/-}$ mice that underwent coronary ligation in addition to preexisting atherosclerosis (resembling more closely the condition in patients with MI), reduction of inflammation improved infarct healing and reduced left ventricular dilation. In conclusion, this work identifies a novel antiinflammatory action of ACE inhibition. The blockade of splenic monocyte release may contribute to the known beneficial effects of ACE inhibitor treatment in patients with MI.
Angiotensin-Converting Enzyme Inhibition Prevents the Release of Monocytes From Their Splenic Reservoir in Mice With Myocardial Infarction

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SUPPLEMENT MATERIAL

ACE inhibition prevents the release of monocytes from their splenic reservoir in mice with myocardial infarction

Leuschner: ACE inhibition impacts monocyte traffic post MI

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Detailed Methods

Animal models
Female C57BL/6J and apoE−/− mice were purchased from Jackson Labs. Cx3cr1gfp/+ mice were obtained by breeding Cx3cr1gfp/gfp mice with C57BL/6J mice. Cx3cr1gfp/+ mice have one Cx3cr1 allele replaced with cDNA encoding eGFP, and can be used to track monocytes 1. ApoE−/− mice had an average age of 45 weeks and were on a high-cholesterol diet (Harlan Teklad, 0.2% total cholesterol), C57BL/6J and Cx3cr1gfp/+ mice were 8-12 weeks old. The institutional animal welfare committee approved the research reported.

MI was induced by permanent coronary ligation as described before 2. Briefly, mice were anesthetized with isoflurane (2% / 2L O2), and intubated and ventilated with an Inspira Advanced Safety Single Animal Pressure/Volume Controlled Ventilator (Harvard Apparatus, Holliston, MA). The chest wall was shaved and left thoracotomy was performed in the 4th left intercostal space. The left ventricle was visualized and the left coronary artery was permanently ligated with monofilament nylon 8-0 sutures (Ethicon, Somerville, NJ) at the site of its emergence from under the left atrium. The chest wall was closed with 7-0 nylon sutures and the skin was sealed with superglue. Splenectomy was done as described previously 3. Mice were treated with a dose of 100 mg/kg 4, 5 Enalapril daily for the acute studies (sacrifice on day 1 after MI) or 20 mg/kg Enalapril daily 6, 7 for the chronic studies. Treatment was started two days before MI and continued for seven days after coronary ligation. In additional cohorts, we initiated Enalapril treatment one hour or twenty-four hours after coronary ligation. Hydralazine was given at a dose of 15 mg/kg 8 daily. Treatment was done by gastric gavage.

Histology
Histology of spleens was assessed for the following groups (n = 5-9 mice per group): wild type C57BL/6J mice and wild type mice 1 day after MI with or without Enalapril treatment. Histology of hearts was assessed for wild type mice 1 day after MI with or without Enalapril treatment. Furthermore, healing was assessed in apoE−/− mice with or without Enalapril treatment on day 4, 7 and 10 after MI. Spleens and hearts were excised, rinsed in PBS and embedded in OCT (Sakura Finetek). Fresh-frozen serial 6 μm thick sections were stained for monocytes and neutrophils (CD11b, BD Pharmingen, NIMP-R14, Santa Cruz Biotechnology), macrophages (MAC-3, BD Pharmingen), neovessels (CD31, BD Pharmingen) and collagen deposition (masson trichrome, Sigma Aldrich). The avidin-biotin peroxidase method was used for immunohistochemistry. The reaction was visualized with a 3-amino-9-athyl-carbazol substrate (AEC, DAKO California). For Immunofluorescence staining sections were incubated with anti-CD11b followed by biotinylated secondary antibody, and texas red-conjugated streptavidin (GE Healthcare). DAPI (Vector Laboratories) was used to identify cell nuclei. Microscopy was done on a Nikon 80i upright fluorescence scope and a Nikon 40 light microscope, both equipped with CCD cameras connected to a Macintosh workstation. Percentage positive area or cell numbers
were quantified using IPLab (version 3.9.3; Scanalytics, Inc., Fairfax, VA) analyzing 5 high
power fields per section and per animal at magnification 200 or 400.

**Flow Cytometry**

Mice were sacrificed on days 1, 2, 3 and 5 after MI and 1 day after ischemia reperfusion injury for flow cytometry (n = 3 – 5 mice per group and time point). Spleens were removed, triturated in HBSS (Mediatech, Inc.) at 4°C with the end of a 3-ml syringe, and filtered through nylon mesh (BD Biosciences). The cell suspension was centrifuged at 300 g for 10 min at 4°C. Red blood cells were lysed with ACK lysis buffer, and the splenocytes were washed with HBSS and resuspended in HBSS supplemented with 0.2% (wt/vol) BSA and 1% (wt/vol) FCS. Heart tissue was harvested, minced with fine scissors, and placed into a cocktail of collagenase I, collagenase XI, DNase I, and hyaluronidase (Sigma-Aldrich) and shaken at 37°C for 1 h. Cells were then triturated through nylon mesh and centrifuged (15 min, 500 g , 4°C). Total spleen and cardiac cell numbers were determined using Trypan blue (Mediatech, Inc.). Peripheral blood was drawn via cardiac puncture with citrate solution (100 mM Na-citrate, 130 mM glucose, pH 6.5), as anti-coagulant and mononuclear cells were purified by density centrifugation. Total blood leukocyte numbers were determined using acetic acid lysis solution (3% HEM A 3 Solution II, 94% ddH 2 O, and 3% glacial acetic acid).

Cell suspensions were incubated with a cocktail of mAbs against T cells (CD90-PE, 53-2.1), B cells (B220-PE, RA3-6B2), NK cells (CD49b-PE, DX5 and NK1.1-PE, PK136), granulocytes (Ly-6G-PE, 1A8), myeloid cells (CD11b-APC, M1/70), antigen-presenting cells (I-A\(^b\) (AF6-120.1)-biotin-Strep-PerCP) and monocyte subsets (Ly-6C-FITC, AL-21) (BD Biosciences). Monocytes were identified as CD11b\(^hi\) (CD90/B220/CD49b/NK1.1/Ly-6G)\(^lo\) (F4/80/I-A\(^b\)/CD11c)\(^lo\) Ly-6C\(^lo\). Macrophages/dendritic cells were identified as CD11b\(^hi\) (CD90/B220/CD49b/NK1.1/Ly-6G)\(^lo\) (F4/80/I-A\(^b\)/CD11c)\(^hi\) Ly-6C\(^lo\). Neutrophils were identified as CD11b\(^hi\) (CD90/B220/CD49b/NK1.1/Ly-6G)\(^hi\) (F4/80/I-A\(^b\)/CD11c)\(^lo\) Ly-6C\(^int\). Reported cell numbers were calculated as the product of total living cells and percent cells within the monocyte/macrophage gate. Within this population, monocyte subsets were identified as (F4/80/I-A\(^b\)/CD11c)\(^lo\) and either Ly-6C\(^hi\) or Ly-6C\(^lo\). Data were acquired on an LSRII (BD Biosciences) and analyzed with FlowJo v.8.5.2 (Tree Star, Inc.).

For analysis by PCR and Western blot, cells were sorted on a BD FACSAria (BD Biosciences). Splenic monocytes were enriched by lineage depletion using MACS LD columns (Miltenyi) and PE–conjugated antibodies against B220, CD49b, NK1.1, Ly-6G, CD90 and Ter-119 followed by anti-PE magnetic beads (Miltenyi). Lineage-depleted cells were further stained with specific antibodies as described above to allow for identification of monocytes.

**ELISA for serum Ang-II levels**

Blood was drawn from mice under anesthesia by cardiac puncture with a syringe pre-loaded with 80 µl of 100 mM EDTA anticoagulant. The blood was transferred to an appendage tube containing 50 µl protease inhibitor cocktail VI (Research Product Inc.), supplemented with 50mM p-hydroxymercuribenzoid acid (Sigma), centrifuged, and supernatant loaded onto Amprep Phenyl PH mini-columns (GE Biosciences) to isolate peptides from the sera. Methanol-eluted peptides were dried by vacuum centrifugation. Ang-II concentration was determined with an Ang-II ELISA (Cayman Chemical), and normalized to the volume of blood samples.

**Intravital microscopy**

During isoflurane anesthesia, the peritoneal cavity was opened with a transverse incision in the disinfected abdominal wall. The gastric-splenic ligament was dissected and the spleen carefully exteriorized. Robust blood flow was observed in the splenic artery during the duration of each experiment and splenic perfusion was confirmed by inspection through fluorescence microscopy upon tail vein injection of an intravascular imaging agent (AngioSense-680, Visen Medical). The exteriorized spleen was completely submerged in temperature-controlled saline solution.
Temperature was carefully monitored using an Omega HH12A thermometer with fine wire thermocouples (Omega Engineering Inc., Stamford, CT) and kept at 37°C.

Images were collected with a prototypical intravital laser scanning microscope (IV100, Olympus Corporation, Tokyo, Japan) using an Olympus 20x UplanFL (NA. 0.5) objective and the Olympus FluoView FV300 version 4.3 program. Samples were excited at 488 nm with an air-cooled argon laser (Melles Griot, Carlsbad, CA) for visualization of GFP+ cells, and at 748 nm with a red diode laser (Model FV10-LD748, Olympus Corporation, Tokyo, Japan) for visualization of the blood pool agent AngioSense-680. Light was collected using custom-built dichroic mirrors SDM-570 and SDM-750, and emission filters BA 505-550 and BA 770 nm IF (Olympus Corporation, Tokyo, Japan). Both channels were collected simultaneously. A prototypical tissue stabilizer (Olympus Corporation, Tokyo, Japan) was used to reduce motion and stabilize the focal plane. The stabilizer was attached to the objective and its z-position was adjusted using a micrometer screw to apply soft pressure on the tissue. Time-lapse recordings were made by collecting images of 256x256 resolution at 15 s intervals over 1 hour in a single plane (2D) of focus. Mice were analyzed in the steady-state or 24 h after MI either treated with Enalapril or untreated.

All GFP+ cells were identified manually in each recording. To determine the displacement over time of individual cells, the centroid position (x-y dimension) of these cells was recorded at the first and last time-point when they could be identified during a recording; then the distance between these two points was calculated, and divided by the elapsed time. Single cell tracks for GFP+ cells were generated based on the position of cell centroids from a series of images recorded at 15 s intervals, and ImageJ and the Manual Tracking plugin (http://rsbweb.nih.gov/ij/plugins/track/track.html) were used for display and quantification. Motion-artifacts in recordings were corrected using the auto-alignment plugin (stackreg) of ImageJ (http://rsb.info.nih.gov/ij/).

In vitro migration
Migration experiments using Ang-II as a chemoattractant were performed in BD BioCoat invasion chambers (BD Bioscience). Sorted monocytes from spleen were suspended in RPMI 1640 media (Cellgro, Mediatech, Inc, VA) supplemented with 0.2% FCS (Valley Biomedical, Inc.); 2x10^5 cells were placed on the matrigel-coated 8 micron pore size PET membrane and incubated in a humidified incubator at 37°C, 5% CO2 for 1h, allowing the cells to attach to the matrigel. For the receptor blocking condition, cells were pretreated with the AT1R antagonist losartan (100 µM) (Merck) for 30 min at 37 C, 5% CO2. Migration was induced by addition of Ang-II (1 µM) (Bachem, Torrance, CA) to the lower compartment. After 2h, non-migrating cells were removed with a cotton tip and the membranes were fixed and stained with Protocol HEMA 3 staining set (Fisher Scientific Company L.L.C.) to identify cells that had migrated to the lower surface of the membrane. The number of migrated cells was determined per x 200 high-power field. Cells that had migrated to the lower chamber were counted using Trypan Blue solution (Cellgro, Mediatech, Inc, VA). Experiments were performed in triplicate.

Western for Ang-II Type 1 (AT1R) receptor on splenic monocytes
Monocytes were isolated by FACS as described above. Sample pellets of ~200,000 monocytes were resuspended in Laemmli buffer (BioRad), and sonicated to lyse cells and shear genomic DNA. Samples were developed by electrophoresis on a 4-15% polyacrilamide gel (BioRad). The proteins were transferred to a polyvinylidene difluoride membrane (Fischer) by semidy transfer. Membranes were blocked with carnation milk and PBS supplemented with 0.05% Tween 20 overnight. Membranes were washed, stained initially with anti-AT1R receptor antibody (Abcam), stripped with Restore buffer (Pierce), and stained with anti-glyceraldehyde-3-phosphate (GAPDH) (Rockland Immunochemicals for Research). Blots were developed with Western Lightning Chemiluminescence reagent (PerkinElmer Instruments) and molecular weights were compared to bands for Precision Plus Protein Western C standards (BioRad).
**Quantitative PCR**

Total mRNA from heart tissue and isolated cells was isolated by Qiagen RNeasy Mini kit and RNeasy Micro kit, respectively. Oligo(dT)-based cDNA was generated by use of the SuperScript® III First-Strand Synthesis kit (Invitrogen), which made cDNA only from the mRNA portion of the total RNA pool. Multiplex quantitative PCR was performed on triplicate samples using Applied Biosystems TaqMan® Assays. Infarct tissue was examined for expression of markers of inflammatory monocytes (Ly-6C), differentiated macrophages (CD68; Mac3), TNF-α, TGF-β, myeloperoxidase, and appropriate controls (GAPDH). Gene expression was determined as x-fold difference after normalizing to GAPDH loading control 10.

**FMT-CT**

On day 1 after MI we performed FMT-CT imaging 11-13 to interrogate the magnitude of inflammation 24 hours after injection of 5 nmol of a pan-cathepsin protease sensor (Prosense-680, PerkinElmer) in apoE⁻/⁻ mice with and without Enalapril treatment and apoE⁻/⁻ mice that were splenectomized at the time of coronary ligation (n = 8-12 per group). A 3D dataset was reconstructed in which fluorescence per voxel was expressed in nM. To robustly identify the region of interest in the heart, anatomic imaging with CT immediately followed free-space FMT at 680/700nm excitation/emission (FMT-2500, Visen Medical). The imaging cartridge containing the anesthetized mouse was placed into the custom machined Plexiglas holder that supplied isoflurane, warm air and optimal positioning in the CT (Inveon PET-CT, Siemens). The CT x-ray source operated at 80 kVp and 500 µA with an exposure time of 370 - 400 ms. The effective 3D resolution was 80 µm isotropic. Isovue-370 was infused intravenously at 55 µL/min through a tail vein catheter. The CT reconstruction protocol performed bilinear interpolation, used a Shepp-Logan filter, and scaled pixels to Hounsfield units. Infarct size was evaluated by iodine-enhanced CT as described before 14. At midventricular level, the enhancing scar and the non-enhancing remote myocardium were tracked manually using OsiriX software (The OsiriX foundation, Geneva, Switzerland). Infarct size was then calculated as percent enhanced area.

**Image fusion**

The fusion method realized three-dimensional mapping of fluorescence within the anatomical reference CT. The approach was based on a multimodality-compatible animal holding device that provides fiducial landmarks on its frame. The imaging cartridge lightly compressed the anesthetized mouse between optically translucent windows and thereby prevented motion during transfer between modalities. The three-dimensional distribution of the fiducials enabled co-registration of datasets in an automated fashion. The point based coregistration tool kit in OsiriX shareware (64 bit, version 3.5.1) fused images after identification of fiducials in respective modalities. Fiducials were tagged with point markers to define their XYZ coordinates. Using these coordinates, data were resampled, rotated and translated to match the image matrices, and finally fused. Fusion was done on a Macintosh computer with a quad-core processor, 16GB RAM and an NVIDIA GeForce graphic card.

**MRI**

We performed in vivo MRI on day 21 after MI in apoE⁻/⁻ mice with and without Enalapril treatment and in apoE⁻/⁻ mice that were splenectomized at the time of coronary ligation (n = 8-12 per group). A 7 Tesla horizontal bore Pharmascan (Bruker) and a custom-made mouse cardiac coil in birdcage design (Rapid Biomedical, Wuerzburg, Germany) was used to obtain cine images of the left ventricular short axis. We employed ECG and respiratory gating using a gradient echo sequence (echo time 2.7 ms, 16 frames per RR interval; flip angle 30 degrees; in-plane resolution 200x200 µm; slice thickness 1 mm). Cardiac volumes were quantitated from 6-8 short axis imaging slices covering the left ventricle as described previously ².
Ischemia reperfusion injury
Ischemia reperfusion injury (IRI) was studied in wild type mice, apoE−/− with and without Enalapril treatment, and in apoE−/− that were splenectomized immediately prior to ischemia. Five minutes after ligation of the coronary artery, 360,000 fluorescent microspheres (10 µm size, excitation/emission wavelength 580/605 nm; Fluospheres, Invitrogen) were slowly injected in a volume of 100 µl into the left ventricle, and distributed to myocardial areas with intact perfusion. Thirty-five minutes after ligation, the suture was released. Twenty-four hours after IRI, 2,3,5-triphenyltetrazolium chloride (TTC, Sigma Aldrich) staining was conducted for evaluation of myocardial infarction and fluorescence reflectance imaging (FRI) to quantify the ischemic area at risk. The heart was harvested, fixed and cut into 1 mm thick myocardial rings. The myocardial rings were scanned using a flat bed scanner (Hewlett Packard). Thereafter, FRI was performed using an epifluorescence microscope (OV110, Olympus). Images were analyzed for TTC-negative infarction area and the ischemic area at risk defined as fluorescence-negative area using Osirix software. Percent infarction over area at risk was calculated as TTC-negative area / FRI-negative area * 100%.

Blood pressure measurements
Blood pressure was monitored in various treatment groups (n = 5-8 per group) with the non-invasive tail cuff CODA System (Kent Scientific) as described previously 15. In mice after coronary ligation, the procedure was done on day 10 after MI.

Statistics
Results are expressed as mean ± SEM. Statistical comparisons between two groups were evaluated by Student’s t-test and corrected by ANOVA for multiple comparisons. A value of p < 0.05 was considered to indicate statistical significance.
Online Figure I: Start of Enalapril one and twenty-four hours after MI
Top: Flow chart illustrating the set up of the study.
A. Mice were started on Enalapril (E) one hour after coronary ligation, and monocyte numbers were analyzed by flow cytometry 24 hours later.
B. Treatment started 24 hours after coronary ligation and was continued for five days. Mean ± SEM; * p < 0.05.
# Online Table I

## Table 1 - Systolic and diastolic blood pressure

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<td>76±4</td>
<td>95±1</td>
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<sup>†</sup> p < 0.05 versus Enalapril, <sup>‡</sup> p < 0.05 vs. Hydralazine, <sup>‡</sup> p < 0.05 vs. MI, Enalapril. Group sizes were n = 5-8. Data are reported as mean ± standard error of mean.
Supplemental References


Legends for the Video file:

Intravital microscopy of monocytic migratory activity in the subcapsular spleen red pulp 1 day after MI

a. The movie shows GFP+ cells (green) in the subcapsular red pulp of a live Cx3cr1<sup>gfp/+</sup> mouse on day 1 after MI. Tracks indicate the path of the centroid of each GFP+ cell at 15 second intervals. Larger steady green cells are dendritic cells / macrophages.

b. GFP+ monocytes in the subcapsular red pulp of the spleen from a Cx3cr1<sup>gfp/+</sup> mouse on day 1 after MI treated with Enalapril. Time is shown in minutes and seconds. Scale bar = 100 µm.

c. and d. The animation shows isolated tracks of monocytes computed from panels a and b.